Blastomyces dermatitidis Antigen-Induced Lymphocyte Reactivity in Human Blastomycosis

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Because of the unavailability of a suitable antigen, specific in vitro lymphocyte reactivity has not been investigated in persons who have had systemic blastomycosis. Twelve persons who had recovered from blastomycosis were evaluated, and all were found to exhibit strongly positive and apparently specific in vitro cellular responses to an alkali-soluble, water-soluble extract of Blastomyces dermatitidis yeast-phase cell walls. Significant lymphocyte transformation induced by this soluble antigen occurred with cells from persons who had recovered from blastomycosis as much as 16 years previously, indicating long-lasting cell-mediated immunity to B. dermatitidis. A simple new in vitro test, lymphocyte migration inhibition, also made it possible to distinguish persons who had had blastomycosis and recovered from persons who had never had the disease. Two in vitro tests of cell-mediated immunity in which the alkali-soluble, water-soluble extract of B. dermatitidis yeast-phase cell walls was employed were highly discriminating. Tests with this antigen may be of value in delineating the epidemiology of blastomycosis and in assessing the prognosis of individual cases.

Tests for evaluating cell-mediated immunity include in vitro assays of lymphocyte blastogenesis and migration inhibition. These tests of cell-mediated immunity have correlated reasonably well with the protection of humans from the progression of fungal infections, including those due to Histoplasma capsulatum (1), Coccidioides immitis (5), Sporothrix schenkii (6), Cryptococcus neoformans (10), and Paracoccidioides brasiliensis (14). The role of cell-mediated immunity in human infection by the dimorphic fungus Blastomyces dermatitidis has been evaluated much less extensively and thus is not well understood (18).

Diminished delayed cutaneous hypersensitivity to blastomycin and decreased blastomycin-induced lymphocyte transformation have been documented in persons evaluated for several months after an epidemic of pulmonary blastomycosis (19). However, because of lack of specificity and relatively low sensitivity, blastomycin has not proved accurate for in vitro assessment of cell-mediated immunity in persons who have had blastomycosis. By using an apparently specific Blastomyces-soluble antigen, we have demonstrated characteristic lymphocyte reactivity in 12 persons who had recovered from endemic blastomycosis.

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MATERIALS AND METHODS

Study population. We evaluated 12 persons from central Tennessee and Kentucky who had recovered from blastomycosis. All had had positive cultures and histological demonstration of the typical yeast forms of B. dermatitidis at the time of diagnosis. They had subsequently been treated with amphotericin B 2 months to 16 years before this study (see Table 1). Antigen specificity was assayed by lymphocyte reactivity to an alkali-soluble, water-soluble extract of B. dermatitidis yeast-phase cell walls (B-ASWS), other soluble fungal antigens, and phytohemagglutinin in these patients and in 11 patients who had systemic histoplasmosis (8 disseminated, 2 chronic pulmonary, and 1 Histoplasma pericarditis) treated from 3 months to 5 years before this study. The same assays were done in six normal adults whose skin tests were positive for histoplasmin and who were residents of Tennessee. Informed consent was obtained from all cell donors.

Antigens. B-ASWS was graciously supplied by R. Cox, San Antonio, Tex. This antigen has been extensively tested in animal models of blastomycosis (7, 11), and its role in stimulating transformation of lymphocytes from histoplasmosis and coccidioidomycosis patients (3) has been evaluated. The dose response of the antigen in those studies indicated that 10 to 50 μg of protein per ml was optimal for lymphocyte trans-

formation and macrophage migration inhibition (3, 7, 11). This was confirmed by the dose response in five B-ASWS-reactive patients (2.5 to 160 μg of protein per ml; 10 μg of protein per ml was the final concentration used in B-ASWS-stimulated cultures). A mycelial cell wall fraction of *B. dermatitidis* (KCB 26) obtained from R. Cox and dialyzed mycelial filtrates (blastomycin) graciously supplied by L. Kaufman, Atlanta, Ga., were likewise evaluated. *H. capsulatum* yeast autolysate prepared in this laboratory essentially by the method of Reeves et al. (17), modified by omitting thimerosal from the refrigerated yeast suspension, was used in a protein concentration of 8.5 μg/ml. *Candida albicans* extract (Hollister-Stier) at a concentration of 12 μg of protein per ml and phytohemagglutinin P (Difco Laboratories; 15.1 μg/ml) were also evaluated.

Lymphocyte transformation. Standard methods were employed for lymphocyte transformation. Briefly heparinized (20 U/ml) peripheral venous blood was separated by Ficoll-Hypaque density gradient centrifugation to yield peripheral blood mononuclear cells (80 to 88% lymphocytes). A total of 5×10^5 cells per ml were suspended in 2 ml of RPMI 1640 medium (Flow Laboratories, Inc.) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.8 U of heparin per ml, and 10% heat-inactivated (56°C), locally prepared, pooled human serum. Parallel cultures with or without antigen were incubated in duplicate at 37°C in 5% CO_2 in humidified air for 5 days. During the final 4 h of culture, a pulse of 2.0 μCi of [^{3}H]thymidine was added, and a harvest was performed with a semiautomated harvester (M24V, Brandel) to collect cells on fiber glass filter pads. Radioactivity was assessed by liquid scintillation counting, with results expressed as the change in disintegrations per minute (Δdpm), calculated by subtracting the disintegrations per minute of antigen-free cells from the disintegrations per minute of antigen-exposed cells. With the exception of one blastomycosis patient with an unstimulated culture count per minute of 8,698, all unstimulated cultures had counts of less than 5,000.

LyMI. Lymphocyte migration inhibition (LyMI) tests were performed as follows. Peripheral blood mononuclear cells (80 to 88% lymphocytes) were adjusted to a concentration of 8×10^6 mixed mononuclear cells per 2 ml of the same medium that was used for transformation, except that heat-inactivated fetal bovine serum was used instead of human serum. Parallel cultures with or without B-ASWS were incubated at 37°C with 5% CO_2 in humidified air for 24 h. We filled 12 capillary tubes (0.8 by 32 mm; Drummond Scientific Co.) by capillary action with antigen-stimulated cell suspensions, and 12 capillary tubes were similarly filled with nonstimulated cell suspensions. After one capillary end was alcohol flame sealed, the suspended cells were centrifuged in a microhematocrit centrifuge for 2 min to yield a cell pellet. Capillary tubes containing either nonstimulated or B-ASWS-stimulated cells were taped to a glass slide and incubated horizontally at 37°C in 5% CO_2 in air. Horizontal locomotion was measured with an ocular micrometer after 48 h of migration. Results were expressed as migration indices, which were calculated by dividing the mean migration distance of the 12 capillary col-

umns of B-ASWS-stimulated cells by the mean migration distance of 12 columns of nonstimulated cells. Direct migration distance was also recorded. Migration measurements at 48 and 72 h after venipuncture gave similar migration indices.

Statistical analysis of data. Lymphocyte transformation and LyMI results were evaluated with the nonparametric Mann-Whitney rank order test.

RESULTS

B-ASWS-induced lymphocyte transformation. Lymphocytes from normal persons were not stimulated by B-ASWS, as indicated by low [^{3}H]thymidine uptake (median Δdpm, 240). Lymphocytes from patients who had recovered from blastomycosis were markedly stimulated by the antigen (median Δdpm, 58,387; $P = 0.0013$) (Fig. 1). Persons who had recovered from histoplasmosis (median Δdpm, 1,057) were also discriminated from blastomycosis patients by their lower lymphocyte transformation responses to B-ASWS ($P = 0.0014$). A statistically significant difference existed between transformation of cells from normal persons and those from histoplasmosis patients by this antigen ($P < 0.04$), but the lymphocytes of both groups underwent B-ASWS-induced [^{3}H]thymidine uptake which was clearly lower than that of cells from blastomycosis patients. The *Blastomyces*
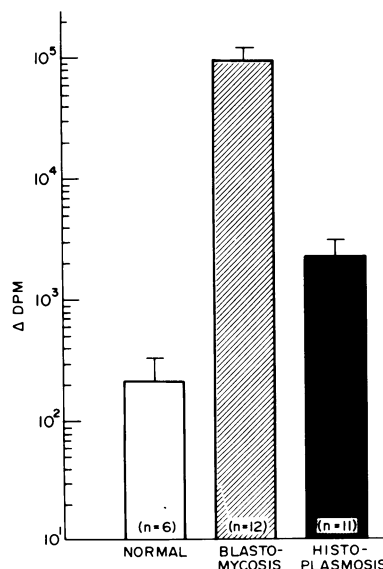

FIG. 1. *Thymidine uptake of cultures containing 10^6 lymphocytes in response to B-ASWS (10 μg/ml). Normal persons were histoplasmin skin test positive. The mean response of lymphocytes from blastomycosis patients is depicted by the central bar. The right-hand bar indicates the responses of cells from persons who had recovered from histoplasmosis. Vertical brackets indicate the SEM.*

cell wall fraction antigen (KCB 26) failed to stimulate lymphocytes from half of the persons who had recovered from blastomycosis (Table 1), and the mycelial filtrate (blastomycin) also failed to do so because it was toxic to the lymphocytes. No correlation between the degree of reactivity and the interval from treatment to subsequent testing was noted for either histoplasmosis patients (data not shown) or blastomycosis patients (Table 1).

Yeast autolysate-induced lymphocyte transformation. Cells from histoplasmin skin test-negative adults did not exhibit significant [^{3}H]thymidine uptake in response to yeast autolysate (median Δdpm, 363), but cells from histoplasmin skin test-positive donors reacted significantly (median Δdpm, 30,905; $P = 0.012$) (Fig. 2). Persons who had histoplasmosis and those who had recovered from blastomycosis had lymphocyte [^{3}H]thymidine uptake that differed significantly ($P = 0.004$) from that of histoplasmin skin test-negative persons. Yeast autolysate-induced transformation of lymphocytes from patients with blastomycosis (median Δdpm, 46,084) resembled that of cells from histoplasmosis patients (median Δdpm, 17,886) and histoplasmin skin test-positive normal persons.

C. albicans antigen-induced transformation. Differences in cellular responses to *C. albicans* antigen did not occur between persons who had recovered from histoplasmosis and those who had recovered from blastomycosis. Mononuclear cells from blastomycosis patients
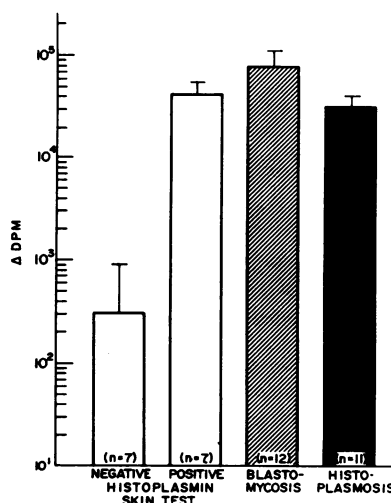

FIG. 2. *Thymidine uptake of cultures containing 10^6 lymphocytes in response to H. capsulatum yeast antigen (yeast autolysate, 8.5 μg/ml). Cells from normal persons responded as indicated by the two bars to the left. Mean responses of cells from persons who had recovered from blastomycosis or histoplasmosis are indicated by the two bars to the right. Vertical brackets display the SEM.*

TABLE 1. *Comparison of [^{3}H]thymidine uptake of lymphocytes from blastomycosis patients induced by B-ASWS or KCB 26*

Patient no.	[^{3}H]thymidine uptake by:		Interval[a]
	B-ASWS	KCB 26	
1	249,243[b] (79.0)[c]	26,270 (9.3)	2 mo
2	10,935 (7.5)	3,566 (3.1)	3 mo
3	57,286 (14.0)	177 (3.1)	9 yr
4	39,219 (9.9)	19,596 (5.0)	16 yr
5	143,243 (26.3)	7,113 (2.3)	8 yr
6	36,664 (13.7)	8,287 (3.9)	8 mo
7	93,927 (26.7)	21,883 (7.0)	9 yr
8	256,594 (111.0)	4,901 (3.1)	2.5 yr
9	31,753 (7.5)	1,723 (1.3)	3 mo
10	20,558 (4.1)	521 (1.1)	10 yr
11	17,969 (7.1)	139 (1.0)	5 yr
12	153,261 (10.8)	76,542 (5.9)	4 yr

[a] Interval between the conclusion of treatment and lymphocyte reactivity assessment.

[b] Δdpm of duplicate samples.

[c] Stimulation indices (in parentheses) were calculated by dividing the disintegrations per minute of antigen-stimulated cultures by the disintegrations per minute of nonstimulated cultures.
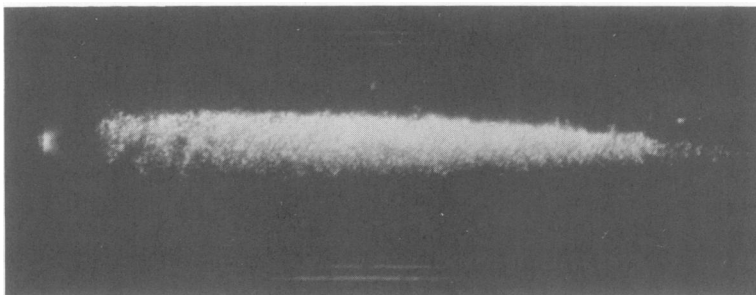
had a mean Δdpm ± standard error of the mean (SEM) of 43,893 ± 14,732, and cells from histoplasmosis patients had a thymidine uptake of 50,442 ± 12,898 mean Δdpm ± SEM.

Phytohemagglutinin-induced transformation. There was no difference in response to phytohemagglutinin between patients who had histoplasmosis and those who had blastomycosis. Thymidine uptake of cells from blastomycosis patients was 252,475 ± 31,345 mean Δdpm ± SEM, and that of cells from histoplasmosis patients was 216,236 ± 23,069 mean Δdpm ± SEM.

B-ASWS-induced LyMI. The mean migration index of mononuclear cells from 6 normal persons was 0.993 (±0.012 SEM), which differed significantly ($P = 0.0009$) from the mean migration index of cells from 12 patients with blastomycosis (0.838 ± 0.016 SEM). Migration indices of less than 0.90 were statistically significant and discriminated normal persons from blastomycosis patients, with one exception. A typical column of migrating cells is shown in Fig. 3. The cells at the apex of the column were lymphocytes, as determined by morphology on Wright-stained smears. Although monocytes were requisite for migration, lymphocytes have made up 90% of the distal two-thirds of the column of migrating cells in previous studies (20). Cells from unstimulated cultures migrated 2.78 ± 0.12 mm, whereas stimulated culture cells from blas-

FIG. 3. *Horizontal intracapillary locomotion from a centrifuged pellet of peripheral blood mononuclear cells seem with a dark-field microscope. The tip of the migrating column was 2.8 mm from the base after 48 h. The distal third of the column of cells was composed almost exclusively of lymphocytes, with monocytes remaining near the proximal end.*

tomycosis patients migrated 2.38 ± 0.127 mm, and B-ASWS-stimulated cells from normal persons migrated 2.79 ± 0.14 mm. Correlation of B-ASWS antigen-induced inhibition of migrating of lymphocytes and their transformation (Fig. 4) demonstrated the superiority of lymphocyte transformation in that the lowest response from blastomycosis patients differed more than 25-fold from the greatest response of normal persons. In contrast, one person with blastomycosis exhibited a negative migration index of 0.94. It would appear that transformation and LyMI discriminate separate lymphocyte functions, as is the case with other lymphocyte reactivity assays (13). For a given blastomycosis patient, stronger migration indices did not correlate exactly with higher Δdpm values. No normal person exhibited an index of less than 0.96. One normal person's migration index of 0.99 and a Δdpm of −1,432 are not included in Fig. 4 because of the negative Δdpm value.

DISCUSSION

Infections with dimorphic fungi such as *H. capsulatum*, *C. immitis*, *S. schenkii*, and *P. brasiliensis* evoke cellular immunity as documented by in vitro evaluation of lymphocyte reactivity (1, 5, 14, 16). In murine models of blastomycosis, a subcutaneous challenge with thimerosal-killed *B. dermatitidis* induced development of delayed hypersensitivity (6), and sublethal infection with the fungus allowed the demonstration of lymphocyte reactivity to specific *Blastomyces* antigens (7, 11). Data from humans correlating infection with the development of specific cell-mediated immunity have been lacking because available *Blastomyces* antigens have not been sufficiently active or specific. Blastomycin, a crude mycelial-phase filtrate, has been used for delayed-hypersensitivity-type skin tests. In two large series, 59 and 100% of patients with positive cultures for *B. dermatitidis* had
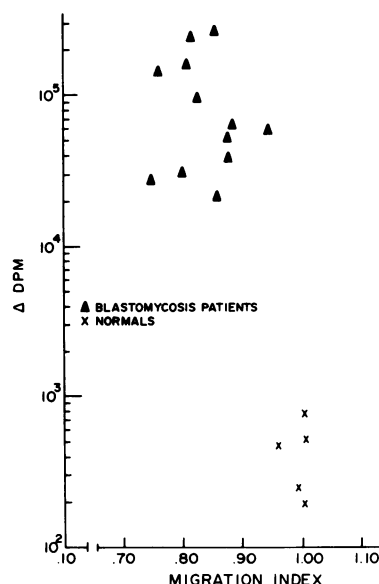

FIG. 4. *Correlation of LyMI and transformation of lymphocytes from normal persons and blastomycosis patients. The values for 1 normal person with a migration index of 0.99 and a Δdpm of −1,432 are not included.*

negative blastomycin skin tests (2, 21), results which could only in part be explained by anergy. In our in vitro studies, blastomycin did not stimulate [³H]thymidine uptake of lymphocyte from blastomycosis patients because of toxicity to the lymphocytes. Due to the lack of a reliable antigen that can be used for skin tests, epidemiological information about blastomycosis has been severely biased through having been derived exclusively from clinical observations. Similar biases affected understanding of the acquisition and pathogenesis of histoplasmosis until standardized histoplasmin became available for accurate skin testing. Only 71 cases of histoplasmosis had been reported by 1945; infection with

H. capsulatum was considered serious and was almost always fatal (15). With the advent of histoplasmin skin testing, it became evident that 80 to 90% of the residents of some parts of the endemic area had become sensitized by previous mild or subclinical infections with *H. capsulatum*.

Serological studies in blastomycosis have likewise been unreliable because of the unavailability of a potent and specific antigen. Serological cross-reactivity has been so common that persons with blastomycosis are just as apt to demonstrate complement-fixing antibodies against histoplasmin as against blastomycin (18).

Responses to blastomycin, as reflected by skin testing and lymphocyte transformation, were observed in 16 persons in an epidemic of pulmonary blastomycosis (18). Of 12 persons with positive lymphocyte transformation in vitro, 10 showed diminished skin test responses and lymphocyte reactivity to blastomycin 36 months after infection. Recently, a more active and specific *Blastomyces* antigen, B-ASWS, has been prepared by Cox and Larsh (4) and by Deighton et al. (7), and it has been useful in evaluating cell-mediated immunity parameters in animals infected with blastomycosis (11). By using B-ASWS we have demonstrated that persons who have recovered from blastomycosis possess persisting specific cell-mediated immune reactivity as measured by lymphocyte transformation or in vitro LyMI assayed up to 16 years after infection. As a precautionary measure, we have initially evaluated only in vitro correlates of human cell-mediated immunity with B-ASWS. However, in animals with blastomycosis, skin testing with B-ASWS correlated well with in vitro lymphocyte reactivity (7); therefore, human delayed-type skin testing with B-ASWS would be the next logical step.

In vitro assessment of cellular immunity to fungal antigens has been complicated by cross-reactions. For example, *C. immitis* and *H. capsulatum* antigens can cross-react in tests of lymphocytes from histoplasmin-sensitive persons (3). Histoplasmin skin test-positive persons exhibit some cross-reactivity to B-ASWS, as measured by lymphocyte transformation, but much less than to KCB 26 (3). In the present study, a significant difference in B-ASWS-induced transformation occurred with cells from patients who had blastomycosis as compared with cells from those who had histoplasmosis, which appears to substantiate a significant degree of antigen specificity. The small difference in B-ASWS-induced lymphocyte transformation between cells from normal persons and those from histoplasmosis patients remains uncertain; the low-level [^{3}H]-thymidine uptake which occurred, though statistically significant, is of uncertain biological importance. Cross-reaction to the *H. capsulatum* antigen by lymphocytes from the patients who had blastomycosis could have occurred to account for the response to yeast autolysate, but that reactivity probably indicated previous asymptomatic *H. capsulatum* infection since all of the patients with blastomycosis were natives of areas of Kentucky and Tennessee wherein approximately 90% of the residents have positive histoplasmin skin tests (9). Blastomycosis patients probably responded to *H. capsulatum* antigen because of prior exposure, just as they responded to *C. albicans* antigen.

LyMI testing correlated well with the standard lymphocyte transformation assay. The patients with blastomycosis provided some measure of the accuracy of this new test. Since there was one patient whose cells failed to demonstrate B-ASWS-induced LyMI, the test did not appear to be as sensitive as transformation. However, no false-positives were detected in normal persons tested. The specificity of LyMI remains to be determined, since histoplasmosis patients were not evaluated with B-ASWS-induced LyMI. To determine the specificity of B-ASWS-induced LyMI, further studies with cells from persons who have recovered from fungal infections other than blastomycosis are necessary.

The LyMI test was performed quite differently from assays of macrophage migrating inhibition factor. Capillary tube migration of lymphocytes (Fig. 3) allows precision in the measurement of lymphocyte locomotion and inhibition by antigen. Relevance for the test may be related to the in vivo phenomenon of retention of lymphocytes in antigen-containing lymph nodes; this sequestration at the site of antigenic stimulation appears to facilitate immune recognition and effector mechanisms (8). Monocytes have been found to be necessary for inhibition of migration, but lymphocytes make up the majority of cells migrating in response to *C. albicans* or tuberculin antigens (12, 20).

Thus, B-ASWS specifically induces lymphocyte transformation in persons who have recovered from blastomycosis. Also, LyMI caused by B-ASWS, apparently by acting on lymphocytes, supports the finding that B-ASWS is sufficiently active for accurate demonstration of in vitro cell-mediated immunity in persons who have recovered from infection with *B. dermatitidis*. Because this reactivity in vitro was shown to be persistent for up to 16 years after treatment, assessment of lymphocyte response to this apparently specific antigen should provide a val-

uable epidemiological tool for assessing the prevalence of immunity to blastomycosis.

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